UTILITY PATENT APPLICATION TRANSMITTAL *(Only for new nonprovisional applications under 37 CFR 1.53(b))*	Attorney Docket No.	0010-1057-0
	First Inventor or Application Identifier	Yoshihiro USUDA, et al.
	Title	METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

09/441055 **11/16/99**

APPLICATION ELEMENTS *See MPEP chapter 600 concerning utility patent application contents*	ADDRESS TO:	Assistant Commissioner for Patents Box Patent Application Washington, DC 20231

1. ■ **Fee Transmittal Form** (e.g. PTO/SB/17) (Submit an original and a duplicate for fee processing)

2. ■ Specification — Total Pages **63**

3. □ Drawing(s) *(35 U.S.C. 113)* — Total Sheets ☐

4. ■ Oath or Declaration — Total Pages **3**
   a. ■ Newly executed (original)
   b. □ Copy from a prior application *(37 C.F.R. §1.63(d))* *(for continuation/divisional with box 15 completed)*
      i. □ DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see *37 C.F.R. §1.63(d)(2) and 1.33(b)*.

5. □ **Incorporation By Reference** *(usable if box 4B is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4B, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

6. □ Assignment Papers (cover sheet & document(s))

7. □ 37 C.F.R. §3.73(b) Statement *(when there is an assignee)* □ Power of Attorney

8. □ English Translation Document *(if applicable)*

9. □ Information Disclosure Statement (IDS)/PTO-1449 □ Copies of IDS Citations

10. □ Preliminary Amendment

11. ■ White Advance Serial No. Postcard

12. □ Small Entity Statement(s) □ Statement filed in prior application. Status still proper and desired.

13. ■ Certified Copy of Priority Document(s) (1) *(if foreign priority is claimed)*

14. ■ Other: Notice of Priority, Receipt of an Original Deposit of Microorganisms for the Purpose of Patent Procedure (FERM BP-6895)

15. If a **CONTINUING APPLICATION**, *check appropriate box, and supply the requisite information below:*
   □ Continuation   □ Divisional   □ Continuation-in-part (CIP) of prior application no.:
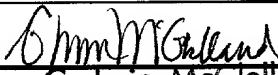   *Prior application information:*   Examiner: Group Art Unit:

16. Amend the specification by inserting before the first line the sentence:
   □ This application is a   □ Continuation   □ Division   □ Continuation-in-part (CIP) of application Serial No.   Filed on
   □ This application claims priority of provisional application Serial No.   Filed

17. CORRESPONDENCE ADDRESS

OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C.
FOURTH FLOOR
1755 JEFFERSON DAVIS HIGHWAY
ARLINGTON, VIRGINIA 22202
(703) 413-3000
FACSIMILE: (703) 413-2220

Name:	Norman F. Oblon	Registration No.:	24,618
Signature:	*C. Irvin McClelland*	Date:	11/16/99
Name:	C. Irvin McClelland Registration Number 21,124	Registration No.:	

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

Technical Field

5   The present invention relates to a method for
producing L-methionine by fermentation. L-methionine is
an important amino acid as a medicament and the like.

Background Art

10

Industrially produced methionine mainly consists
of DL-methionine, which is produced through chemical
synthesis. When L-methionine is required, it is
provided through production of N-acetyl-DL-methionine by
15   acetylation of DL-methionine and subsequent enzymatic
selective deacetylation of the N-acetylated L-methionine.
On the other hand, as for the production of L-
methionine by fermentation, methods utilizing an L-
methionine analogue-resistant mutant strain have been
20   reported. However, their production amount is small,
and factors affecting the L-methionine production have
not been elucidated yet. Therefore, L-methionine is
still one of the amino acids the most difficult to be
produced by fermentation. For example, while methods
25   utilizing *Escherichia coli* (*E. coli*) K-12 strain have
been reported in Japanese Patent Laid-open (Kokai) No.
56-35992 and literature (Chattapadhyay, M.K. *et al.*, *Med.*

Sci. Res. 23, 775 (1995); Chattapadhyay, M.K. et al.,
Biotechnol. Lett. 17, 567-570 (1995)), any of these
methods cannot provide L-methionine production amount
sufficient for industrial use.

5   In *E. coli,* the biosynthetic pathway of L-
methionine is partly shared with the biosynthetic
pathway of L-threonine, and L-homoserine serves as a
common intermediate. The first step of the peculiar
pathway from L-homoserine to L-methionine is catalyzed
10   by homoserine transsuccinylase (HTS). This enzyme has
been known to suffer concerted inhibition by the final
product, L-methionine, and a metabolite of L-methionine,
S-adenosylmethionine (Lee, L.-W. et *al., J. Biol. Chem.,*
241, 5479-5480 (1966)).

15   The nucleotide sequence of the *metA* gene encoding
homoserine transsuccinylase of *E. coli,* has been
reported by Duclos et al. (Duclos, B. *et al., Nucleic*
Acids Res. 17, 2856 (1989)), and a method for obtaining
a strain having a mutation for *metA* using resistance to
20   an analog of L-methionine, α-methyl-DL-methionine (MM)
has also been known (Chattopadhyay, M.K. et *al., J. Gen.*
Microbiol., 137, 685-691 (1991)). It has been reported
for *Salmonella typhimurium* that, the *metA* gene product,
homoserine transsuccinylase, was an inhibition-
25   desensitized type as for the inhibition by L-methionine
and S-adenosylmethionine (SAM) in an MM resistant strain
(Lawrence, D.A. et *al., J. Bacteriol., 109,* 8-11 (1972)).

However, the nucleotide sequence of the mutant *metA* gene has not been reported. Furthermore, it has been reported that a mutant having a sole mutation in *metA* did not secret L-methionine (Chattopadhyay, M.K. *et al.*,

5 *J. Gen. Microbiol.*, *137*, 685-691 (1991)).

It has also been revealed that the expression of the genes including *metA* of the enzymes for the reaction by homoserine transsuccinylase and subsequent reactions in the peculiar biosynthetic pathway of L-methionine

10 suffers inhibition by a repressor which is a *metJ* gene product (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed. Neidhardt, F.D., ASM Press, pp.542-560 (1996)). It has also been known that the

15 *metJ* gene is adjacent to the *metBL* operon in a reverse direction, which operon consists of the *metB* gene coding for the second enzyme of the peculiar biosynthetic pathway for L-methionine, cystathionine γ-synthase, and metL coding for aspartokinase-homoserine dehydrogenase

20 II (AK-HDII) (Duchange, N. *et al.*, *J. Biol. Chem.*, *258*, 14868-14871 (1983)).

It has been suggested that *metK* coding for S-adenosylmethionine, which catalyzes the metabolic reaction from L-methionine to S-adenosylmethionine,

25 should be an essential enzyme (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed.

Neidhardt, F.D., ASM Press, pp.542-560 (1996)).
Furthermore, it has also been known that a *metJ* mutant
strain can be obtained based on resistance to a
methionine analogue such as DL-norleucine and ethionine

5   (Chattopadhyay, M.K. *et al.*, *J. Gen. Microbiol.*, *137*,
685-691 (1991)), and can increase the expression of the
enzymes of the peculiar biosynthetic pathway of L-
methionine (Greene, R.C. *et al.*, *J. Bacteriol.*, *115*, 57-
67).

10   As mentioned above, there have been reported
enzymes involved in the L-methionine biosynthesis and
genes therefor to some extent. However, only few
findings that directly lead to the production of L-
methionine by fermentation have been obtained, and hence

15   hardly applied to breeding of L-methionine-producing
bacteria.

Summary of the Invention

20   The present invention has been accomplished in
view of the aforementioned current technical status, and
an object of the present invention is to elucidate
factors affecting the L-methionine production, thereby
breeding L-methionine-producing bacteria, and enabling

25   L-methionine production by fermentation.

In order to achieve the aforementioned object, the
present inventors earnestly conducted studies, and as a

result, accomplished the present invention.

That is, the present invention provides:

(1)　A microorganism which is deficient in repressor of L-methionine biosynthesis system and has L-methionine productivity;

(2)　a microorganism having enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;

(3)　a microorganism which is deficient in repressor of L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;

(4)　the microorganism according to any one of the above (1)-(3), which further exhibits reduced intracellular S-adenosylmethionine synthetase activity;

(5)　the microorganism according to any one of the above (2)-(4), wherein the enhanced intracellular homoserine transsuccinylase activity is obtained by increasing copy number of a gene encoding homoserine transsuccinylase, or enhancing an expression regulatory sequence for the gene;

(6)　the microorganism according to the above (1) or (4), which has homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized;

(7)　the microorganism according to any one of the above (1)-(6), which exhibits L-threonine

auxotrophy;

(8)   the microorganism according to any one of the above (1)-(7), which exhibits enhanced intracellular cystathionine γ-synthase activity and enhanced

5   intracellular aspartokinase-homoserine dehydrogenase II activity;

(9)   the microorganism according to any one of the above (1)-(8), which belongs to the genus *Escherichia*;

10   (10)   a method for producing L-methionine which comprises culturing the microorganism according to any one of the above (1)-(9) in a medium to produce and accumulate L-methionine in the medium, and collecting the L-methionine from the medium; and

15   (11)   A DNA which codes for homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized, wherein the homoserine transsuccinylase has the amino acid sequence of SEQ ID NO: 26 including a mutation

20   corresponding to replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of proline by leucine at the 298th position, mutation corresponding to

25   replacement of arginie by cysteine at the 27th position and replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of

isoleucine by serine at the 296th position and
replacement of proline by leucine at the 298th position,
mutation corresponding to replacement of proline by
leucine at the 298th position and replacement of

5   arginine by cysteine at the 27th position, or mutation
corresponding to replacement of arginine by cysteine at
the 27th position, replacement of isoleucine by serine
at the 296th position and replacement of proline by
leucine at the 298th position.

10   In this specification, S-adenosylmethionine will
occasionally be abbreviated as "SAM", α-methyl-DL-
methionine as "MM", and DL-norleucine as "NL". Further,
S-adenosylmethionine synthetase will be occasionally be
abbreviated as "SAM synthetase", and homoserine

15   transsuccinylase as "HTS". The metB gene product,
cystathionine γ-synthase, of E. coli may also be called
as "cystathionine synthase", and the metL gene product,
"aspartokinase homoserine dehydrogenase II", may also be
called as AK-HDII.

20   The term "L-methionine productivity" used for the
present invention means an ability to accumulate L-
methionine in a medium when a microorganism is cultured
in the medium.

According to the present invention, there is

25   provided a microorganism having L-methionine production
ability. The microorganism can be utilized as an L-
methionine-producing bacterium or a material for

breeding of L-methionine-producing bacteria.

The mutant *metA* gene of the present invention can be utilized for the breeding of L-methionine-producing bacteria, because the concerted inhibition by L-

5   methionine and SAM for the enzyme encoded by it is canceled.

Detailed Description of the Invention

10   Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a microorganism which is deficient in repressor of the L-methionine biosynthesis system and has L-methionine

15   productivity, or a microorganism which has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity. The microorganism of the present invention is preferably a microorganism which is deficient in repressor of the L-methionine biosynthesis

20   system, and has enhanced intracellular homoserine transsuccinylase activity. The microorganism of the present invention further preferably exhibits reduced intracellular SAM synthetase activity.

The aforementioned microorganism of the present

25   invention is not particularly limited, so long as it has a pathway for producing L-methionine and SAM from L-homoserine via O-acylhomoserine which is produced from

L-homoserine by the acyl-transferring reaction, and its

expression of the acyl transferase is controlled through

suppression by a repressor. While such a microorganism

may be an *Escherichia* bacterium, coryneform bacterium,

5   and Bacillus bacterium, it is preferably an *Escherichia*

bacterium, for example, *E. coli.*

If the microorganism of the present invention is a

bacterium in which HTS possessed by the microorganism

suffers concerted inhibition by SAM and L-methionine

10 like *E. coli*, its L-methionine productivity may be

improved by canceling the inhibition.

As the peculiar pathway for the methionine

biosynthesis, there are one which uses cystathionine as

an intermediate as in many microorganisms such as *E.*

15 *coli*, and one which does not use cystathionine as in

Brevibacterium flavum (Ozaki, H. *et al.*, *J. Biochem.*, *91*,

1163 (1982)). In the present invention, a microorganism

that uses cystathionine is preferred. In such a

microorganism, L-methionine productivity can be

20 increased by enhancing the intracellular cystathionine

synthase activity. In addition, even if it is a

microorganism like *Brevibacterium flavum*, L-methionine

productivity may be enhanced by deficiency of repressor

in the L-methionine biosynthesis system and/or

25 enhancement of HTS.

Furthermore, in the aforementioned microorganism,

L-methionine productivity can further be increased by

enhancing at least one of the aspartokinase activity and the homoserine dehydrogenase activity, which are involved in the shared pathway of the L-methionine biosynthesis and the L-threonine biosynthesis.

5   When two or more of the aforementioned characteristics are imparted to a microorganism, the order for imparting them is not particularly limited, and they can be given in an arbitrary order. Moreover, when multiple genes are introduced into a microorganism,

10   those genes may be carried by the same vector, or may be separately carried by multiple different vectors. When multiple vectors are used, it is preferred to use vectors having different drug markers and different replication origins.

15   Methods for imparting each of the aforementioned characteristics to a microorganism will be explained below.

<1> Deficiency of repressor in L-methionine biosynthesis

20   system

A microorganism deficient in a repressor in the L-methionine biosynthesis system can be obtained by subjecting microorganisms to a mutagenic treatment, and selecting a strain no longer producing the repressor.

25   The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used

for mutagenesis such as N-methyl-N'-nitrosoguanidine
(NTG) and nitrous acid.

The repressor can also be made deficient by
destroying a gene coding for the repressor on

5   chromosomal DNA of the microorganism. The gene can be
destroyed by preparing a deleted type gene which has
deletion of at least a part of a coding region or
expression regulatory sequence, and causing homologous
recombination of the deleted type gene and a gene on the

10 chromosome to substitute the deleted type gene for the
gene on the chromosome (gene substitution).

Since the nucleotide sequence of the gene coding
for the repressor in the L-methionine biosynthesis
system of *E. coli* (*metJ*) has been known (Duchange, N. *et*

15 *al., J. Biol. Chem., 258*, 14868-14871 (1983)), the
repressor can be isolated from chromosomal DNA, for
example, by PCR using primers produced based on the
nucleotide sequence. A deleted type gene can be
obtained by excising a certain region from the gene

20 fragment obtained as described above, and deleting at
least a part of the coding region or expression
regulatory region.

The gene substitution can be performed, for
example, as follows. A deleted type gene is introduced

25 into a vector having a temperature sensitive replication
origin to prepare a recombinant vector, and a
microorganism is transformed with the recombinant vector

so that the deleted type gene should be inserted into a gene on chromosomal DNA by homologous recombination of the deleted type gene and the gene on the chromosomal DNA. Then, the transformant strain is cultured at a

5   temperature at which the vector cannot replicate to drop out the vector from cytoplasm. Furthermore, the gene is replaced when one copy of the gene on the chromosome is dropped out with the vector. The occurrence of the desired gene substitution can be confirmed by Southern

10 hybridization analysis of the chromosomal DNA of a strain to be to be tested for the gene substitution.

As a vector for *E. coli* that has a temperature sensitivity replication origin, for example, the plasmid pMAN997 disclosed in Japanese Patent Application No. 9-

15 194603 and the like can be mentioned. As a vector for coryneform bacteria that has a temperature sensitivity replication origin, for example, the plasmid pHSC4 disclosed in Japanese Patent Laid-open No. 5-7491 and the like can be mentioned. However, the vector is not

20 limited to these, and other vectors can also be used.

As mentioned above, it has known for *E. coli* that the *metJ* gene is adjacent to the *metBL* operon, which consists of *metB* gene and *metL* gene, in the reverse direction (Duchange, N. *et al.*, *J. Biol. Chem.*, *258*,

25 14868-14871 (1983)). Therefore, if a suitable promoter sequence is ligated to a deleted type *metJ* gene and gene substitution is performed as described above, the

destruction of the *metJ* gene and improvement of

expression utilizing substitution of promoter in the

metBL operon can simultaneously be obtained by one

homologous recombination. Improved expression of the

5 *metBL* operon enhances the intracellular cystathionine

synthase activity and AK-HDII activity.

Specifically, the following three components, a

fragment of about 1 kb containing the metB gene, which

is obtained by, for example, PCR (polymerase chain

10 reaction; White, T. J. *et al.*; *Trends Genet.*, *5*, 185

(1989)) utilizing chromosomal DNA of *E. coli* W3110

strain as a template, and oligonucleotides having the

nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 6 as

primers; a fragment of about 1 kb containing the

15 downstream region of the *metJ* gene obtained by PCR

utilizing oligonucleotides having the nucleotide

sequences of SEQ ID NO: 7 and SEQ ID NO: 8 as primers;

and a sequence having a promoter sequence of the

threonine operon, which is obtained by annealing of the

20 oligonucleotides represented as SEQ ID NO: 9 and SEQ ID

NO: 10, can be inserted into a suitable vector, and

ligating to it to obtain a recombinant vector which

contains a DNA fragment having deletion of the

structural gene of *metJ* and substitution of threonine

25 promoter for the promoter of the *metBL* operon.

To introduce the recombinant DNA prepared as

described above to bacterium, any known transformation

methods can be employed. For instance, employable are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for Escherichia coli K-12 [see Mandel, M.

5 and Higa, A., *J. Mol. Biol.*, *53*, 159 (1970)]; and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for Bacillus subtilis [see Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene, 1,*

10 153 (1977)]. Alternatively, it is also possible to apply a method in which DNA recipient cells are allowed to be in a state of protoplasts or spheroplasts capable of incorporating recombinant DNA with ease to introduce recombinant DNA into the DNA recipient cells, as known

15 for Bacillus subtilis, actinomycetes, and yeasts (Chang, S. and Choen, S. N., *Molec. Gen. Genet.*, *168*, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., *Nature, 274*, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., *Proc. Natl. Acad. Sci. USA, 75*, 1929

20 (1978)). Transformation of coryneform bacteria may performed by the electric pulse method (refer to Japanese Patent Publication Laid-Open No. 2-207791).

The vector to be used for cloning the genes such as *metA*, *metK* and *thrBC* as described below includes, for

25 example, pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, RSF1010 and the like. Besides, it is possible to use phage DNA vectors. When microorganisms other than *E.*

coli are used, it is preferable to use a shuttle vector

autonomously replicable in those microorganisms and *E.*

coli. As examples of plasmid autonomously replicable in

coryneform bacteria, for example, the followings can be

5   mentioned.

pAM330 (see Japanese Patent Laid-open No. 58-

67699)

pHM1519 (see Japanese Patent Laid-open No. 58-

77895)

10   pAJ655 (see Japanese Patent Laid-open No. 58-

192900)

pAJ611 (see the same)

pAJ1844 (see the same)

pCG1   (see Japanese Patent Laid-open No. 57-

15   134500)

pCG2   (see Japanese Patent Laid-open No. 58-

35197)

pCG4   (see Japanese Patent Laid-open No. 57-

183799)

20   pCG11   (see the same)

pHK4   (see Japanese Patent Laid-open No. 5-7491)

In order to prepare recombinant DNA by ligating

the gene fragment and a vector, the vector is digested

by restriction enzyme(s) corresponding to the termini of

25   the gene fragment. Ligation is generally performed by

using a ligase such as T4 DNA ligase.

The methods to perform, for example, preparation

of the genomic DNA library, hybridization, PCR,

preparation of plasmid DNA, digestion and ligation of

DNA, and design of oligonucleotide used for primers are

described by Sambrook, J., Fritsche, E. F., Maniatis, T.

5   in Molecular Cloning, Cold Spring Harbor Laboratory

Press (1989).

<2> Enhancement of HTS activity and introduction of

mutant HTS

10   The HTS activity in a microbial cell can be

attained by preparing a recombinant DNA through ligation

of a gene fragment encoding HTS with a vector which

functions in the microorganism, preferably a multi-copy

type vector, and transforming the microorganism through

15   introduction of the plasmid into in the microbial cell.

As a result of increase of the copy number of the gene

encoding HTS in the transformant strain, the HTS

activity is enhanced. In *E. coli*, HTS is encoded by the

metA gene. When an *Escherichia* bacterium is used as the

20   microorganism, the HTS gene to be introduced is

preferably a gene derived from an *Escherichia* bacterium.

However, genes derived from other microorganisms having

homoserine transacetylase, such as coryneform bacteria,

can also be used.

25   Enhancement of HTS activity can also be achieved

by introducing multiple copies of the HTS gene into the

chromosomal DNA of the above-described host strains. In

order to introduce multiple copies of the HTS gene in the chromosomal DNA of bacterium belonging to the genus *Corynebacterium*, the homologous recombination is carried out using a sequence whose multiple copies exist in the

5   chromosomal DNA as targets. As sequences whose multiple copies exist in the chromosomal DNA, repetitive DNA, inverted repeats exist at the end of a transposable element can be used. Also, as disclosed in Japanese Patent Laid-open No. 2-109985, it is possible to

10   incorporate the HTS gene into transposon, and allow it to be transferred to introduce multiple copies of the HTS gene into the chromosomal DNA. By either method, the number of copies of the HTS gene within cells of the transformant strain increases, and as a result, HTS

15   activity is enhanced.

The enhancement of HTS activity can also be obtained by, besides being based on the aforementioned gene enhancement, enhancing an expression regulatory sequence for the HTS gene. Specifically, it can be

20   attained by replacing an expression regulatory sequence of HTS gene on chromosome DNA or plasmid, such as a promoter, with a stronger one (see Japanese Patent Laid-open No. 1-215280). For example, lac promoter, trc promoter, tac promoter, PR promoter and PL promoter of

25   lambda phage and the like are known as strong promoters. Substitution of these promoters enhances expression of the HTS gene, and hence the HTS activity is enhanced.

Since the nucleotide sequence of the HTS gene (metA) of *E. coli* has been known (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)), it can be isolated from chromosomal DNA by PCR using primers produced based on

5   the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 21 and SEQ ID NO: 22 are specifically mentioned.

It is expected that, by enhancing the HTS activity

10   in a microbial cell as described above, L-methionine biosynthesis can be enhanced, and thus the L-methionine production amount can be increased.

Further, because HTS suffers concerted inhibition by L-methionine and SAM, the L-methionine biosynthesis

15   system can also be enhanced by obtaining a microorganism containing HTS for which concerted inhibition has been canceled. Such a microorganism containing HTS for which concerted inhibition has been canceled can be obtained by subjecting microorganisms to a mutagenic treatment,

20   and selecting a strain containing HTS for which concerted inhibition has been canceled. The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used for mutagenesis such as

25   N-methyl-N'-nitrosoguanidine (NTG) and nitrous acid. The expression "HTS for which concerted inhibition has been canceled" herein used means HTS exhibiting a ratio

of its enzymatic activity in the presence of L-
methionine, SAM or L-methionine and SAM to its enzymatic
activity in the absence of L-methionine and SAM
(remaining ratio) higher than that of a wild-type HTS.

5   Specifically, an HTS exhibiting a remaining ratio in the
presence of 1 mM L-methionine of 40% or more, preferably
80% or more, a remaining ratio in the presence of 1 mM
SAM of 10% or more, preferably 50% or more, or a
remaining ratio in the presence of 0.1 mM each of L-

10   methionine and SAM of 15% or more, preferably 60% or
more is an HTS for which concerted inhibition by L-
methionine and SAM has been canceled.

A mutant strain having such a mutant HTS as
mentioned above can be obtained by culturing a parent

15   strain in the presence of α-methyl-DL-methionine (MM),
e.g., in a medium containing 1 g/l of MM, and selecting
a strain growing on the medium. The selection with MM
may be repeated two or more times.

A mutant strain having a mutant HTS can also be

20   obtained by cloning the mutant HTS gene (mutant *metA*)
from a HTS mutant obtained as described above, and
transforming a microorganism with the mutant gene.
Isolation of a mutant HTS gene and introduction of the
gene into a microorganism can be performed as the

25   aforementioned wild-type HTS gene. As HTS having a
mutant *metA* gene, HTS having the amino acid sequence of
SEQ ID NO: 26 including a mutation corresponding to

replacement of arginine by cysteine at the 27th position,
mutation corresponding to replacement of isoleucine by
serine at the 296th position, or mutation corresponding
to replacement of proline by leucine at the 298th

5   position can specifically be mentioned. HTS including
two or more of these mutations is also a preferred
mutant HTS.

<3> Attenuation of SAM synthetase activity

10   Furthermore, the L-methionine productivity of
microorganism can be increased by attenuating
intracellular SAM synthetase activity. The L-methionine
productivity of a microorganism can also be increased by
making the microorganism SAM synthetase activity

15   deficient, but in such a case, the medium for culturing
the microorganism must contain SAM. Therefore, it is
preferable to attenuate SAM synthetase activity. The
expression "attenuating SAM synthetase activity" herein
used means that a specific activity of SAM synthetase

20   per unit of protein in microbial cells is made lower
than that of a strain having a wild-type SAM synthetase.
Specifically, the degree of attenuation, i.e., the
reduced activity may be 80% to 50%, preferably 50% to
30%, more preferably 30% to 10% of the SAM synthetase

25   activity of a wild-type strain. In *E. coli*, it has been
suggested that, if the specific activity of SAM
synthetase falls below 10%, cell division would be

inhibited (Newman, E. B. *et al.*, *J. Bacteriol.*, 18Q, 3614-3619 (1998)).

The microorganism whose SAM synthetase activity is reduced may be one producing SAM synthetase exhibiting a reduced specific activity per enzymatic protein (reduced type SAM synthetase), or one in which expression efficiency of the enzyme is reduced because of reduced transcription efficiency or reduced translation efficiency of SAM synthetase gene.

A mutant strain whose SAM synthetase activity is reduced may be obtained by culturing a parent strain in the presence of DL-norleucine (NL), e.g., in a medium containing 0.1 g/l of NL, and selecting a grown strain. The selection with NL may be repeated two or more times. It is also possible to use ethionine or γ-glutamylmethyl ester instead of DL-norleucine.

A mutant strain having an reduced type SAM synthetase can also be obtained by cloning a gene for the reduced type SAM synthetase from a SAM synthetase-reduced strain obtained as described above, and substituting the mutant gene for a wild-type SAM synthetase gene on a chromosome of microorganism. The gene substitution of SAM synthetase gene can be performed in the same manner as the aforementioned *metJ* gene. Since the nucleotide sequence of the SAM synthetase gene (*metK*) of *E. coli* has been known (Blattner, F.R. *et al.*, *Science*, 277, 1453-1462 (1997)),

it can be isolated from chromosomal DNA by PCR using primers produced based on the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 11 and SEQ ID NO: 12

5   are specifically mentioned. The occurrence of the desired mutation in the obtained *metK* gene can be confirmed by determining the nucleotide sequence of the gene, and comparing it with a known nucleotide sequence of wild-type *metK* gene.

10   As specific examples of the gene coding for an reduced type SAM synthetase, those coding for SAM synthetases having the amino acid sequence of SEQ ID NO: 18 including a mutation corresponding to replacement of isoleucine by leucine at the 303rd position, mutation

15   corresponding to replacement of valine by glutamic acid at the 185th position, or mutation corresponding to replacement of arginine at the 378th position and subsequent amino acid residues by a sequence of alanine-methionine-leucine-proline-valine (SEQ ID NO: 29) can be

20   mentioned.

<4> L-Threonine auxotrophy

L-methionine productivity can be improved by imparting L-threonine auxotrophy to a microorganism.

25   Specific examples of a microorganism exhibiting L-threonine auxotrophy include those having deficiency of any one of enzymes involved in the peculiar pathway of

L-threonine biosynthesis from L-homoserine to L-
threonine. In *E. coli*, the genes of the enzymes
involved in the biosynthesis of L-threonine exist as the
threonine operon (*thrABC*), and L-threonine auxotrophic

5 strain, which has lost the ability to synthesize L-
homoserine and subsequent products, can be obtained by
deleting the *thrBC* segment. The *thrA* gene codes for one
of the isozymes of aspartokinase, which is an enzyme of
the shared pathway of the L-methionine and L-threonine

10 biosyntheses, and hence it is preferably not to be
deleted.

In order to delete *thrBC*, the *thrBC* segment in the
threonine operon on chromosomal DNA can be destroyed.
thrBC can be destroyed by replacing the *thrBC* segment on

15 a microbial chromosome with *thrBC* a part of which is
deleted. The gene substitution of *thrBC* may be
performed in the same manner as in the gene substitution
of the aforementioned *metJ* gene. A *thrBC* segemnt
containing deletion can be obtained by amplifying a

20 fragment of about 1 kb containing the upstream region of
the *thrB* gene by PCR using *E. coli* chromosomal DNA as a
template and primers having the nucleotide sequences of
SEQ ID NOS: 1 and 2, similarly amplifying a fragment of
about 1 kb containing the downstream region of the *thrC*

25 gene by PCR using primers having the nucleotide
sequences of SEQ ID NOS: 3 and 4, and ligating these
amplified products.

<5> Production of L-methionine

L-methionine can be produced by culturing a microorganism having L-methionine productivity prepared as described above in a medium so that L-methionine should be produced and accumulated in the medium, and collecting L-methionine from the medium.

The medium to be used may be selected from well-known media conventionally used depending on the kind of microorganism to be used. That is, it may be a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic ingredients as required. Any special medium is not needed for the practice of the present invention.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B1, L-threonine and L-tyrosine or yeast extract to be contained in appropriate amounts as

organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

5   Cultivation is preferably carried out under an aerobic condition for 16-120 hours. The cultivation temperature is preferably controlled at 25°C to 45°C, and pH is preferably controlled at 5-8 during cultivation. Inorganic or organic, acidic or alkaline

10   substances as well as ammonia gas or the like can be used for pH adjustment.

Any special techniques are not required for collecting L-methionine from the medium after the cultivation in the present invention. That is, the

15   present invention can be practiced by a combination of well-known techniques such as techniques utilizing ion exchange resin and precipitation.

Best Mode for Carrying out the Invention

20

The present invention will further be explained more specifically with reference to the following examples.

25   Example 1: Acquisition of L-threonine auxotrophic strain and metJ deficient strain from Escherichia coli W3110 strain

<1> Preparation of plasmid for recombination containing *thrBC* structural gene having deletion

Chromosomal DNA was prepared from W3110 strain,

5   which was a derivative of the wild-type K-12 strain of *E. coli*, by using a genomic DNA purification kit (Advanced Genetic Technology) according to the instruction of the kit. Oligonucleotides having the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 in Sequence Listing

10   were synthesized. PCR was performed according to the method of Erlich *et al.* (PCR Technology-Principles and Applications for DNA Amplification, ed. Erlich, H.A., Stockton Press) by using the above oligonucleotides as primers and the aforementioned chromosomal DNA as the

15   template to amplify a fragment of about 1 kb containing the upstream region of the *thrB* gene. This amplification fragment was introduced with recognition sequences for *Eco*RI and *Sal*I, which were derived from the primers. The obtained amplified fragment was

20   digested with restriction enzymes corresponding to the introduced recognition sites.

Similarly, PCR was performed by using oligonucleotides having the nucleotide sequences of SEQ ID NO: 3 and SEQ ID NO: 4 as primers to amplify a

25   fragment of about 1 kb containing the downstream region of the *thrC* gene. This amplification fragment was introduced with recognition sequences for *Sal*I and

*Hind*III, which were derived from the primers. The
obtained amplified fragment was digested with
restriction enzymes corresponding to the introduced
recognition sites. The aforementioned two amplified

5 fragments, and pHSG398 (TAKARA SHUZO) digested with
*Eco*RI and *Hind*III were ligated by using a ligation kit
(TAKARA SHUZO), and *E. coli* JM109 competent cells
(TAKARA SHUZO) were transformed with the ligation
product. Plasmids were prepared from the transformants

10 based on the alkaline method (Boirnboim, H.C. *et al.*,
Nucleic Acids Res., 7, 1513-1523 (1979)) by using a
Plasmid Extractor PI-50 (Kurabo Industries, Ltd.). From
the obtained plasmids, a plasmid in which two fragments
were inserted in the *Eco*RI and *Hind*III recognition sites

15 through *Sal*I recognition sites was selected based on the
lengths of inserted fragments. This plasmid contained
the upstream and the downstream regions of the
structural gene of *thrBC*, and contained a gene fragment
in which substantially full length of the structural

20 gene of *thrBC* was deleted.

<2> Production of *thrBC* structural gene deletion strain
by genetic recombination

    The aforementioned plasmid and the plasmid pMAN997

25 having a temperature sensitive replication origin, which
was disclosed in Japanese Patent Application No. 9-
194603, were digested with *Eco*RI and *Hind*III, and

ligated each other. The *E. coli* JM109 strain was

transformed with the obtained recombinant plasmid.

Plasmids were extracted from the transformants, and one

having a structure where a *thrBC*-deleted gene fragment

5   was inserted in pMAN997 was selected, and designated as

pMANΔBC. The W3110 strain was transformed with this

plasmid to perform genetic recombination in a

conventional manner. That is, selection of recombinant

strains was carried out based on the L-threonine

10   auxotrophy in M9 medium (Sambrook, J. et *al.*, "Molecular

Cloning: A Laboratory Manual/Second Edition", Cold

Spring Harbor Laboratory Press, A.3 (1989)), and the

obtained L-threonine auxotrophic strain was designated

as WΔBC strain.

15

<3> Production of *metJ* deficient strains from W3110

strain and WΔBC strain

Then, PCR was performed by using the W3110 strain

chromosomal DNA as the template and oligonucleotides

20   having the nucleotide sequences of SEQ ID NO: 5 and SEQ

ID NO: 6 as primers to amplify a fragment of about 1 kb

containing the *metB* gene. This amplification fragment

was introduced with recognition sequences for EcoRI and

SphI. The obtained amplified fragment was digested with

25   restriction enzymes corresponding to the introduced

recognition sites.

Similarly, PCR was performed by using

oligonucleotides having the nucleotide sequences of SEQ
ID NO: 7 and SEQ ID NO: 8 as primers to amplify a
fragment of about 1 kb containing the downstream region
of the *metJ* gene. This amplification fragment was

5   introduced with recognition sequences for *SalI* and
HindIII. The obtained amplified fragment was digested
with restriction enzymes corresponding to the introduced
recognition sites.

Then, a sequence represented as SEQ ID NO: 9,

10   which contained SphI and *HindIII* recognition sites at
the both ends and the promoter sequence of the threonine
operon, and its complementary strand represented as SEQ
ID NO: 10 were synthesized, annealed, and digested with
restriction enzymes *SphI* and *HindIII*. The threonine

15   promoter fragment obtained as described above, pHSG298
(TAKARA SHUZO) digested with *EcoRI*, and the
aforementioned two PCR amplification fragments were
mixed, and ligated. The JM109 strain was transformed
with this ligation solution, and plasmids were extracted

20   from the transformants. A plasmid comprising ligated
four of the components was selected from the obtained
plasmids. This plasmid had a structure where the *metJ*
structural gene was deleted, and the promoter of *metBL*
operon was replaced with the threonine promoter.

25   The plasmid obtained above and the plasmid pMAN997
having a temperature sensitive replication origin, which
is disclosed in Japanese Patent Application No. 9-194603,

were digested with *EcoRI*, and ligated each other. A plasmid having a structure where a *metJ*-deleted fragment was inserted into pMAN997 was selected, and designated as pMANΔJ. The W3110 strain and the WΔBC strain were

5 transformed with this plasmid to perform genetic recombination in a conventional manner. Selection of recombinant strains was performed based on the lengths of amplified products from PCR utilizing DNA prepared from the cells as the template, and the oligonucleotides

10 represented in SEQ ID NO: 6 and SEQ ID NO: 8 as primers. The *metJ*-deleted strains obtained from the W3110 strain and the WΔBC strain were designated as WΔJ strain and WΔBCΔJ strain, respectively.

In order to confirm the effect of the *metJ*

15 deletion by the recombination, a crude enzyme extract was prepared from the cells, and the activities of HTS and cystathionine synthase were measured. The W3110 strain and the WΔJ strain were each inoculated to 2 ml of LB medium, and cultured at 37°C overnight. 1 ml of

20 the medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The obtained cell were suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of Davis-Mingioli minimal medium (Davis B.D., and Mingioli, E.S., *J.*

25 *Bacteriol.*, *60*, 17-28 (1950)) containing 5 mM L-methionine. The cells were cultured at 37°C for 24 hours, then the medium was centrifuged at 8,000 rpm for

10 minutes, and the cells were washed twice with 0.9%

saline. The cells were suspended in 3 ml of 50 mM

potassium phosphate buffer (pH 7.5) containing 1 mM

dithiothreitol. This suspension was subjected to a cell

5   disruption treatment at 4°C with a power of 150 W for 5

minutes by using an ultrasonicator (Kubota Co.). The

sonicated suspension was centrifuged at 15,000 rpm for

30 minutes, and the supernatant was desalted in a

Sephadex G-50 column (Pharmacia) to obtain a crude

10   enzyme extract. The HTS activity and the cystathionine

synthase activity in the crude enzyme extract were

measured.

As for the HTS activity, 5 μl of the crude enzyme

extract was added to a reaction mixture comprising 0.1 M

15   potassium phosphate (pH 7.5), 1 mM succinyl-coenzyme A

(Sigma), 0.2 nM DL-[^{14}C]homoserine (Muromachi Chemical

Industry), and 0.2 mM L-homoserine to obtain a volume of

50 μl, and allowed to react at 30°C for 10 minutes. 1

μl of the reaction mixture was spotted on a cellulose

20   plate (Merck), and developed with a mixed solvent

containing acetone, butanol, water, and diethylamine at

a ratio of 10:10:5:2. After the plate was air-dried,

autoradiography was performed by using an image analyzer

(Fuji Photo Film).

25   The cystathionine synthase has been known to

produce α-ketobutyric acid, ammonia, and succinic acid

from O-succinylhomoserine in the absence of L-cysteine,

and this can be utilized for simple detection thereof (Holbrook, E.L. *et al.*, *Biochemistry*, *29*, 435-442 (1990)). 100 μl of the crude enzyme extract was added to a reaction mixture comprising 0.2 M Tris-HCl (pH 8),

5 5 mM O-succinylhomoserine (Sigma), and 0.25 mM pyridoxal phosphate (Sigma) to obtain a volume of 1 ml, allowed to react at 37°C for 20 minutes, and cooled with ice. The O-succinylhomoserine in this reaction mixture was quantitated by reverse phase HPLC (GL Sciences), and the

10 reduced amount of O-succinylhomoserines was calculated by using a reaction mixture not added the crude enzyme extract. The reaction was also performed with no addition of pyridoxal phosphate, and pyridoxal phosphate-dependent reduction of O-succinylhomoserine

15 was defined to be the cystathionine synthase activity.

The specific activities for the HTS activity and the cystathionine synthase activity measured as described above were shown in Table 1. While the HTS activity was hardly detected in the W3110 strain due to

20 the effect of L-methionine addition, marked activity was observed in the WΔJ strain. As also for the cystathionine synthase activity, remarkable increase was observed in the WΔJ strain compared with the W3110 strain. From these results, the effects of the *metJ*

25 deletion and the promoter substitution in the *metBL* operon by recombination were confirmed.

Table 1: HTS activity and cystathionine synthase

activity in metJ deficient strain

Strain	HTS activity (mmol/min/mg protein)	Cystathionine synthase activity (mmol/min/mg protein)
W3110	0.3	140
WΔJ	126	1300

Example 2: Acquisition of metK mutant from W3110 strain

The W3110 strain was cultured in LB medium

(Sambrook, J. et al., "Molecular Cloning: A Laboratory

Manual/Second Edition", Cold Spring Harbor Laboratory

Press, A.1 (1989)) at 37°C overnight. 1 ml of the

cultured medium was centrifuged at 5,000 rpm for 10

minutes, and the cells were washed twice with 0.9%

saline. The obtained cells were suspended in 100 μl of

0.9% saline, 10 μl of which was inoculated to 5 ml of

Davis-Mingioli minimal medium containing 0.1 g/l of DL-

norleucine (NL), and cultured at 37°C for 5 days.

Some of the grown colonies were subjected to

colony separation on LB agar medium, and their growth

was confirmed again in Davis-Mingioli minimal medium

containing 0.1 g/l of NL to select 12 NL-resistant

strains. Chromosomal DNA was prepared from these

resistant strains. PCR was performed by using this

chromosomal DNA as a template and two sorts of primers

having the sequences of SEQ ID NOS: 11 and 12 to amplify

the metK gene. The nucleotide sequence of this

amplification fragment was determined by using

amplification primers of which sequences are shown as

SEQ ID NO: 11 and 12, and primers of which sequences are

shown as SEQ ID NOS: 13, 14, 15, and 16. The nucleotide

5   sequence determination was performed by using a Dye

Terminator Cycle Sequencing Kit (Perkin-Elmer) on a DNA

sequencer Model 373S (Perkin-Elmer) in accordance with

the instructions attached to them. The nucleotide

sequence of the wild strain W3110 determined as a

10   control completely coincided with the sequence of *metK*

reported by Blattner et al. (Blattner, F.R. et *al.*,

Science, 277, 1453-1462 (1997)). This sequence is

represented as SEQ ID NO: 17. Further, the amino acid

sequence of SAM synthetase which may be encoded by the

15   sequence of SEQ ID NO: 17 is shown in SEQ ID NO: 18.

Among the NL resistant strains, a mutation in the

structural gene of *metK* was found in 3 strains out of

the 12 strains, which were designated as WNL2, WNL24,

and WNL32. As for the *metK* nucleotide sequences of

20   these mutant strains, in the wild-type nucleotide

sequence shown as SEQ ID NO: 17, the WNL2 strain had

replacement of adenine by cytosine at the 907th position,

the WNL24 strain had replacement of thymine by adenine

at the 554th position, and the WNL32 strain had deletion

25   of cytosine at the 1132nd position. As a result, it was

found that, in the amino acid sequence of SAM synthetase

represented as SEQ ID NO: 18, the SAM synthetase of the

WNL2 strain had replacement of isoleucine by leucine at

the 303rd position, that of the WNL24 strain had

replacement of valine by glutamic acid at the 185th

position, and that of the WNL32 strain had replacement

5   of arginine at the 378th position and subsequent amino

acid residues by alanine-methionine-leucine-proline-

valine due to deletion of one nucleotide. It was

estimated that the SAM synthetase activity was reduced

in these strains.

10

Example 3: Production of L-methionine by introduction of

metK mutation and amplification of wild-type metA gene

(1) Introduction of metK mutation into WΔBCΔJ strain

15   PCR was performed by using each chromosomal DNA of

the metK gene mutant strains, WNL2 strain, WNL24 strain,

and WNL32 strain, as a template, and oligonucleotides of

SEQ ID NO: 19 and SEQ ID NO: 20 as primers to amplify a

fragment of about 2.5 kb containing the metK gene. This

20   amplification fragment was introduced with recognition

sequences for HindIII at the both ends. The obtained

amplified fragment was digested with HindIII. pSTV28

(TAKARA SHUZO) digested with HindIII and the PCR

amplification fragment were mixed and ligated, and the

25   JM109 strain was transformed with the ligation product.

Plasmids were extracted from the transformants. From

the obtained plasmids, plasmids inserted with the PCR

amplification fragment were selected. As for these plasmids, mutations in the *metK* structural gene were confirmed by determining their nucleotide sequences.

HindIII digestion fragments of these plasmids were

5   each cloned into pMAN997 digested with *Hind*III, and the resulting plasmids were designated as pMANK-2, pMANK-24, and pMANK-32, respectively. The WΔBCΔJ strain was transformed with these plasmids to obtain genetic recombination in a conventional manner. Chromosomal DNA

10 was extracted from the recombinant strains, and used as a template together with oligonucleotides having the nucleotide sequences of SEQ ID NO: 11 and SEQ ID NO: 12 as primers to perform PCR, and the amplification products were examined for nucleotide sequence to select

15 those having mutations. The *metK* mutant strains obtained from the WΔBCΔJ strain were designated as WΔBCΔJK-2 strain, WΔBCΔJK-24 strain, and WΔBCΔJK-32 strain, respectively.

20 (2) Amplification of *metA* gene

PCR was performed by using W3110 strain chromosomal DNA as a template, and oligonucleotides having the nucleotide sequences of SEQ ID NO: 21 and SEQ ID NO: 22 as primers to amplify a fragment of about 1 kb

25 containing the *metA* gene. This amplification fragment was introduced with recognition sequences for *Sph*I and *Sal*I at the both ends. The obtained amplified fragment

was digested with restriction enzymes corresponding to
the introduced recognition sites. The digested product
was cloned into pHSG398 digested with *SphI* and *SalI*.
The nucleotide sequence of the inserted fragment was

5   determined by using amplification primers represented as
SEQ ID NOS: 21 and 22, and primers having the sequences
of SEQ ID NOS: 23 and 24. The determined nucleotide
sequence of *metA* of the wild strain W3110 completely
coincided with the sequence of *metA* reported by Blattner

10   *et al.* (Blattner, F.R. *et al.*, *Science*, 277, 1453-1462
(1997)). This sequence is represented as SEQ ID NO: 25.
Further, the amino acid sequence of HTS which may be
encoded by the sequence of SEQ ID NO: 25 is shown as SEQ
ID NO: 26.

15   A *SphI* and *SalI* digestion product of this plasmid,
HindIII and *SphI* digestion product of the threonine
promoter of Example 1, and pMW118 (Nippon Gene) digested
with *HindIII* and *SalI* were mixed and ligated. The JM109
strain was transformed with this reaction mixture, and

20   plasmids were extracted from the transformants. From
the obtained plasmids, a plasmid in which the three
components were ligated was selected. This plasmid had
a structure where the *metA* gene was positioned at the
downstream from the threonine promoter, by which the

25   *metA* was expressed. This plasmid was designated as
pMWPthmetA-W. The W3110 strain, WΔBC strain, WΔBCΔJ
strain, WΔBCΔJK-2 strain, WΔBCΔJK-24 strain, and

WΔBCΔJK-32 strain were transformed with this plasmid to
obtain transformants.

Each transformant was cultured at 37°C overnight
on an LB plate containing 50 mg/l of ampicillin. The
5   cells were inoculated to 20 ml of medium at pH 7
containing 40 g/l of glucose, 1 g/l of magnesium sulfate,
16 g/l of ammonium sulfate, 1 g/l of potassium
dihydrogenphosphate, 2 g/l of yeast extract (Bacto
Yeast-Extract, Difco), 0.01 g/l of manganese sulfate,
10   0.01 g/l of iron sulfate, 30 g/l of calcium carbonate,
50 mg/l of ampicillin, and 0.5 g/l of L-threonine, and
cultured at 37°C for 48 hours.

The cells were separated from the culture, and the
amount of L-methionine was measured by an amino acid
15   analyzer (Hitachi). The results are shown in Table 2.
L-Methionine, which was not detected for the W3110
strain, was increased in the WΔBC strain and the WΔBCΔJ
strain. Concerning the *metK* mutant strains, while the
amount of L-methionine decreased in the WΔBCΔJK-2 strain,
20   a comparable amount was observed in the WΔBCΔJK-32
strain, and the amount was increased in the WΔBCΔJK-24
strain. Thus, the effect on the L-methionine production
was observed. The WΔBCΔJK-24 strain harboring the
plasmid pMWPthrmetA-W was given a private number AJ13425,
25   and it was deposited at the National Institute of
Bioscience and Human-Technology, Agency of Industrial
Science and Technology, Ministry of International Trade

and Industry (postal code 305-8566, 1-3 Higashi 1-chome,
Tsukuba-shi, Ibaraki-ken, Japan) on May 14, 1998 as an
accession number of FERM P-16808, and transferred from
the original deposit to international deposit based on

5   Budapest Treaty on September 27, 1999, and has been
deposited as deposition number of FERM BP-6895.

Table 2: L-methionine production amount of

wild-type *metA* introduced strains

Strain	Production amount (g/l)
W3110/pMWPthrmetA-W (*metA*ᵉ)	0.000
WΔBC/pMWPthrmetA-W (*thrBC⁻*, *metA*ᵉ)	0.008
WΔBCΔJ/pMWPthrmetA-W (*metBL*ᵉ, *thrBC⁻*, *metA*ᵉ)	0.022
WΔBCΔJK-2/pMWPthrmetA-W (*thrBC⁻*, *metJ⁻*, *metBL*ᵉ, *metK¹*, *metA*ᵉ)	0.014
WΔBCΔJK-24/pMWPthrmetA-W (*thrBC⁻*, *metJ⁻*, *metBL*ᵉ, *metK¹*, *metA*ᵉ)	0.141
WΔBCΔJK-32/pMWPthrmetA-W (*thrBC⁻*, *metJ⁻*, *metBL*ᵉ, *metK¹*, *metA*ᵉ)	0.023

10   *metK¹*: reduced *metK*, *metA*ᵉ: enhanced *metA*,

*metBL*ᵉ: enhanced *metBL*

Example 4: Acquisition of *metA* mutant strain and inhibition-desensitized type *metA* gene

15

The W3110 strain was inoculated to 2 ml of LB
medium, and cultured at 37°C for 8 hours. 1 ml of the
medium was centrifuged at 5,000 rpm for 10 minutes, and
the cells were washed twice with 0.9% saline. The

20   obtained cells were suspended in 100 μl of 0.9% saline,

5 μl of which was inoculated to 5 ml of Davis-Mingioli
minimal medium containing 1 g/l of α-methyl-DL-
methionine (MM), and cultured at 37°C for 3 days. The
medium was properly diluted, plated on Davis-Mingioli

5   minimal medium containing 1 g/l of MM, and cultured at
37°C overnight. Some of grown colonies were subjected
to colony separation on LB agar medium, and their growth
was confirmed again on Davis-Mingioli minimal medium
containing 1 g/l of MM. This procedure was

10 independently performed 9 times, and six independent
resistant strains, each designated as WMM4, WMM5, WMM6,
WMM7, WMM8, and WMM9, were obtained.

Chromosomal DNA was prepared from these resistant
strains. PCR was performed by using each chromosomal

15 DNA as a template and primers having the sequences
represented as SEQ ID NOS: 21 and 22 to amplify the metA
gene. The nucleotide sequence of each amplification
fragment was determined by using primers for
amplification of which nucleotide sequences are

20 represented as SEQ ID NOS: 21 and 22, and primers having
the sequences represented as SEQ ID NOS: 23 and 24. As
for the metA nucleotide sequence of the resistance
strains, in the nucleotide sequence of wild-type metA
represented as SEQ ID NO: 25, thymine at the position of

25 887 was changed to guanine in the WMM4 strain, cytocine
at the position of 893 was changed to thymine in the
WMM5 strain, was the wild-type sequence was found in the

WMM6 strain, a sequence of ATCTC corresponding the 886th to the 890th nucleotides iterated twice, and an insertion sequence consisting of about 1300 nucleotides, called IS2 (Ghosal, D. *et al.*, *Nucleic Acids Res.*, *6*, 1111-1122 (1979)), was present between the repeated sequences in the WMM7 and WMM8 strains, and cytosine at the position of 79 was changed to thymine in the WMM9 strain. As a result, it was found that, in the amino acid sequence of HTS represented as SEQ ID NO: 26, the 296th isoleucine was changed to serine in the WMM4 strain, proline at the position of 298 was changed to leucine in the WMM5 strain, proline at the position of 298 and subsequent amino acid residues were changed to a sequence of arginine-leucine-alanine-proline due to an insertion sequence in the WMM7 and WMM8 strains, and arginine at the position of 27 was changed to cysteine in the WMM9 strain.

The strains WMM4, WMM5, WMM9 and WMM7, in which a mutation was observed in the *metA* structural gene, were each cultured in LB medium contained in a test tube at 37° overnight, and 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes. The cells were washed twice with 1 ml of 0.9% saline, and suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of minimal medium and cultured at 37°C for one day. The medium was centrifuged at 8,000 rpm for 10 minutes, and the cells were washed twice with 1 ml of 0.9% saline.

The obtained cells were suspended in 3 ml of 50 mM
potassium phosphate buffer (pH 7.5) containing 1 mM
dithiothreitol, and a crude enzyme extract was obtained
in the same manner as in Example 1. The HTS activity in

5   the crude enzyme extract was measured with the reaction
composition mentioned in Example 1 in the presence of an
inhibitor. The results are shown in Table 3. The
activity was undetectable for the WMM7 strain, and this
was considered to reflect the marked decrease of the

10   specific activity due to the amino acid sequence change
caused by the insertion sequence. The specific
activities of the other strains were about 1/4 of the
wild strain. The inhibition by MM was canceled in all
of the WMM4, WMM5, and WMM9 strains, and the inhibition

15   by L-methionine was also reduced considerably. While
the inhibition by SAM was hardly canceled in the WMM9
strain, tendency of cancellation was observed in the
WMM4 and WMM5 strains. Inhibition by the combination of
L-methionine and SAM, which exhibited the strongest

20   inhibition for the wild-type HTS activity, was also
markedly reduced in the WMM4 and WMM5 strains

25

Table 3: Activity of HTS derived from MM resistant

strains in the presence of various inhibitors

Inhibitor	HTS activity (mmol/min/mg protein)				
	W3110	WMM9	WMM4	WMM5	WMM7
No addition	22.3	5.0	4.5	4.5	0.0
0.1 mM MM	18.6	4.9	4.1	4.6	0.0
1 mM MM	7.0	2.7	4.6	4.8	0.0
0.1 mM Met	14.3	2.5	4.5	4.2	0.0
1 mM Met	0.8	2.2	4.0	4.0	0.0
0.1 mM SAM	17.0	1.1	4.6	3.6	0.0
1 mM SAM	3.0	0.5	2.6	3.3	0.0
0.1 mM SAM + 0.1 mM Met	0.0	0.9	5.6	2.8	0.0

Example 5: L-Methionine production by

introduction of mutant metA

PCR was performed by using chromosomal DNA from
each of the WMM9, WMM4 and WMM5 strains among the *metA*
mutants obtained in Example 4 as a template, and
oligonucleotides having the sequences of SEQ ID NO: 21
and SEQ ID NO: 22 as primers to amplify a fragment
containing the *metA* gene. This amplification fragment
had recognition sequences for *Sph*I and *Sal*I at the both
ends. The both ends of this amplified fragment were
digested with *Sph*I and *Sal*I, and cloned into pHSG398
digested with *Sph*I and *Sal*I. The nucleotide sequence of
each insert fragment was determined to confirm the
mutation point. A *Sph*I and *Sal*I digestion product of
this plasmid, *Hind*III and *Sph*I digestion product of the
threonine promoter mentioned in Example 1, and pMW118
(Nippon Gene) digested with *Hind*III and *Sal*I were mixed

and ligated. The JM109 strain was transformed with this ligation solution, and plasmids were extracted from the transformants. From the obtained plasmids, those comprising ligated three components were selected, and

5   designated as pMWPthrmetA-9, pMWPthrmetA-4, and pMWPthrmetA-5, respectively.

Further, in order to obtain combination of the mutation points of each mutant *metA* gene, site-specific mutagenesis was performed by using Mutan-Super Express

10   Km (TAKARA SHUZO) according to the instruction of the manufacturer. pMWPthrmetA-9+4 were produced by combining the *metA-4* mutation with the *metA-9* mutation using an oligonucleotide having the sequence of SEQ ID NO: 27. pMWPthrmetA-9+5 was similarly produced by

15   combining the *metA-5* mutation with the *metA-9* mutation. Furthermore, pMWPthrmetA-9+4+5 was produced by combining the *metA-9* mutation with the *metA-4* and *metA-5* mutations using an oligonucleotide having the sequence of SEQ ID NO: 28.

20   The WΔBCΔJK-32 strain was transformed with these plasmids to obtain transformants. Each of the transformants was cultured at 37°C overnight on an LB plate containing 50 mg/l of ampicillin. The cells were inoculated to 20 ml of medium at pH 7 containing 40 g/l

25   of glucose, 1 g/l of magnesium sulfate, 16 g/l of ammonium sulfate, 1 g/l of potassium dihydrogenphosphate, 2 g/l of yeast extract (Bacto Yeast-Extract, Difco),

0.01 g/l of manganese sulfate, 0.01 g/l of iron sulfate,

30 g/l of calcium carbonate, 50 mg/l of ampicillin, and

0.5 g/l of L-threonine, and cultured at 37°C for 48

hours. The cells were separated from the culture, and

5 the amount of L-methionine was measured by an amino acid

analyzer (Hitachi). The results are shown in Table 4.

The amount of L-methionine accumulation increased

several times in the strains introduced with a mutant

metA, compared with the strain introduced with a wild-

10 type *metA*. Furthermore, by combining mutations, further

increase of L-methionine production amount was obtained.

Table 4: L-methionine production amount of mutant

metA-introduced strains

Strain	Amount of L-methionine production (g/l)
WΔBCΔΔJK-32/pMWPthrmetA-W	0.023
WΔBCΔJK-32/pMWPthrmetA-9	0.158
WΔBCΔJK-32/pMWPthrmetA-4	0.108
WΔBCΔJK-32/pMWPthrmetA-5	0.131
WΔBCΔJK-32/pMWPthrmetA-9+4	0.206
WΔBCΔJK-32/pMWPthrmetA-5+9	0.207
WΔBCΔJK-32/pMWPthrmetA-9+4+5	0.236

15

SEQUENCE LISTING

<110> USUDA, Yoshihiro
      KURAHASHI, Osamu

<120> Method for Producing L-Methionine by Fermentation

<130> OP914

<140>
<141> 1999-11-

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<160> 29

<170> PatentIn Ver. 2.0

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<213> Artificial Sequence

<220>
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<210> 2
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
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<400> 2

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<210> 3
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 3
gggtcgacat cagtaaaatc tattcatt   28

<210> 4
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 4
ggaagcttgc ccgagggaaa gatctgta   28

<210> 5
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 5
gggcatgccc agggaacttc atcacatg   28

<210> 6
<211> 28
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 6
gggaattctc atggttgcgg cgtgagag   28

<210> 7
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 7
ggaagcttgc gtgagatggg gattaacc   28

<210> 8
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 8
gggaattcta ctgctagctg ctcttgcg   28

<210> 9
<211> 75
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

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cacagacgca tgccc   75

<210> 10
<211> 75

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 10
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aaattttaag cttcc   75

<210> 11
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
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<400> 11
caacagtttg agctaacc   18

<210> 12
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 12
gcggtttttt tgccggatgc   20

<210> 13
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 13

tcggctacgc aactaatg   18

<210> 14
<211> 18
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<400> 14
gagaatgcac cgccaccg   18

<210> 15
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<220>
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<400> 15
tggcgcgtca cggtggcg   18

<210> 16
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 16
gcacgtcggt ttcattag   18

<210> 17
<211> 1155
<212> DNA
<213> Escherichia coli

<220>

<221> CDS
<222> (1)..(1152)

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Met Ala Lys His Leu Phe Thr Ser Glu Ser Val Ser Glu Gly His Pro
 1               5                  10                  15

gac aaa att gct gac caa att tct gat gcc gtt tta gac gcg atc ctc    96
Asp Lys Ile Ala Asp Gln Ile Ser Asp Ala Val Leu Asp Ala Ile Leu
                20                  25                  30

gaa cag gat ccg aaa gca cgc gtt gct tgc gaa acc tac gta aaa acc   144
Glu Gln Asp Pro Lys Ala Arg Val Ala Cys Glu Thr Tyr Val Lys Thr
            35                  40                  45

ggc atg gtt tta gtt ggc ggc gaa atc acc acc agc gcc tgg gta gac   192
Gly Met Val Leu Val Gly Gly Glu Ile Thr Thr Ser Ala Trp Val Asp
        50                  55                  60

atc gaa gag atc acc cgt aac acc gtt cgc gaa att ggc tat gtg cat   240
Ile Glu Glu Ile Thr Arg Asn Thr Val Arg Glu Ile Gly Tyr Val His
 65                  70                  75                  80

tcc gac atg ggc ttt gac gct aac tcc tgt gcg gtt ctg agc gct atc   288
Ser Asp Met Gly Phe Asp Ala Asn Ser Cys Ala Val Leu Ser Ala Ile
                85                  90                  95

ggc aaa cag tct cct gac atc aac cag ggc gtt gac cgt gcc gat ccg   336
Gly Lys Gln Ser Pro Asp Ile Asn Gln Gly Val Asp Arg Ala Asp Pro
                100                 105                 110

ctg gaa cag ggc gcg ggt gac cag ggt ctg atg ttt ggc tac gca act   384
Leu Glu Gln Gly Ala Gly Asp Gln Gly Leu Met Phe Gly Tyr Ala Thr
            115                 120                 125

aat gaa acc gac gtg ctg atg cca gca cct atc acc tat gca cac cgt   432
Asn Glu Thr Asp Val Leu Met Pro Ala Pro Ile Thr Tyr Ala His Arg
            130                 135                 140

ctg gta cag cgt cag gct gaa gtg cgt aaa aac ggc act ctg ccg tgg   480
Leu Val Gln Arg Gln Ala Glu Val Arg Lys Asn Gly Thr Leu Pro Trp
145                 150                 155                 160

ctg cgc ccg gac gcg aaa agc cag gtg act ttt cag tat gac gac ggc   528
Leu Arg Pro Asp Ala Lys Ser Gln Val Thr Phe Gln Tyr Asp Asp Gly
                165                 170                 175

aaa atc gtt ggt atc gat gct gtc gtg ctt tcc act cag cac tct gaa   576
Lys Ile Val Gly Ile Asp Ala Val Val Leu Ser Thr Gln His Ser Glu
                180                 185                 190

gag atc gac cag aaa tcg ctg caa gaa gcg gta atg gaa gag atc atc   624
```

Glu Ile Asp Gln Lys Ser Leu Gln Glu Ala Val Met Glu Glu Ile Ile
       195   200   205

aag cca att ctg ccc gct gaa tgg ctg act tct gcc acc aaa ttc ttc   672
Lys Pro Ile Leu Pro Ala Glu Trp Leu Thr Ser Ala Thr Lys Phe Phe
   210   215   220

atc aac ccg acc ggt cgt ttc gtt atc ggt ggc cca atg ggt gac tgc   720
Ile Asn Pro Thr Gly Arg Phe Val Ile Gly Gly Pro Met Gly Asp Cys
225   230   235   240

ggt ctg act ggt cgt aaa att atc gtt gat acc tac ggc ggc atg gcg   768
Gly Leu Thr Gly Arg Lys Ile Ile Val Asp Thr Tyr Gly Gly Met Ala
          245   250   255

cgt cac ggt ggc ggt gca ttc tct ggt aaa gat cca tca aaa gtg gac   816
Arg His Gly Gly Gly Ala Phe Ser Gly Lys Asp Pro Ser Lys Val Asp
          260   265   270

cgt tcc gca gcc tac gca gca cgt tat gtc gcg aaa aac atc gtt gct   864
Arg Ser Ala Ala Tyr Ala Ala Arg Tyr Val Ala Lys Asn Ile Val Ala
          275   280   285

gct ggc ctg gcc gat cgt tgt gaa att cag gtt tcc tac gca atc ggc   912
Ala Gly Leu Ala Asp Arg Cys Glu Ile Gln Val Ser Tyr Ala Ile Gly
   290   295   300

gtg gct gaa ccg acc tcc atc atg gta gaa act ttc ggt act gag aaa   960
Val Ala Glu Pro Thr Ser Ile Met Val Glu Thr Phe Gly Thr Glu Lys
305   310   315   320

gtg cct tct gaa caa ctg acc ctg ctg gta cgt gag ttc ttc gac ctg 1008
Val Pro Ser Glu Gln Leu Thr Leu Leu Val Arg Glu Phe Phe Asp Leu
          325   330   335

cgc cca tac ggt ctg att cag atg ctg gat ctg ctg cac ccg atc tac 1056
Arg Pro Tyr Gly Leu Ile Gln Met Leu Asp Leu Leu His Pro Ile Tyr
          340   345   350

aaa gaa acc gca gca tac ggt cac ttt ggt cgt gaa cat ttc ccg tgg 1104
Lys Glu Thr Ala Ala Tyr Gly His Phe Gly Arg Glu His Phe Pro Trp
          355   360   365

gaa aaa acc gac aaa gcg cag ctg ctg cgc gat gct gcc ggt ctg aag 1152
Glu Lys Thr Asp Lys Ala Gln Leu Leu Arg Asp Ala Ala Gly Leu Lys
   370   375   380
taa 1155

<210> 18
<211> 384
<212> PRT
<213> Escherichia coli

<400> 18

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 1               5                   10                  15
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            20                  25                  30
Glu Gln Asp Pro Lys Ala Arg Val Ala Cys Glu Thr Tyr Val Lys Thr
        35                  40                  45
Gly Met Val Leu Val Gly Gly Glu Ile Thr Thr Ser Ala Trp Val Asp
        50                  55                  60
Ile Glu Glu Ile Thr Arg Asn Thr Val Arg Glu Ile Gly Tyr Val His
65                  70                  75                  80
Ser Asp Met Gly Phe Asp Ala Asn Ser Cys Ala Val Leu Ser Ala Ile
            85                  90                  95
Gly Lys Gln Ser Pro Asp Ile Asn Gln Gly Val Asp Arg Ala Asp Pro
            100                 105                 110
Leu Glu Gln Gly Ala Gly Asp Gln Gly Leu Met Phe Gly Tyr Ala Thr
            115                 120                 125
Asn Glu Thr Asp Val Leu Met Pro Ala Pro Ile Thr Tyr Ala His Arg
            130                 135                 140
Leu Val Gln Arg Gln Ala Glu Val Arg Lys Asn Gly Thr Leu Pro Trp
145                 150                 155                 160
Leu Arg Pro Asp Ala Lys Ser Gln Val Thr Phe Gln Tyr Asp Asp Gly
                165                 170                 175
Lys Ile Val Gly Ile Asp Ala Val Val Leu Ser Thr Gln His Ser Glu
            180                 185                 190
Glu Ile Asp Gln Lys Ser Leu Gln Glu Ala Val Met Glu Glu Ile Ile
            195                 200                 205
Lys Pro Ile Leu Pro Ala Glu Trp Leu Thr Ser Ala Thr Lys Phe Phe
            210                 215                 220
Ile Asn Pro Thr Gly Arg Phe Val Ile Gly Gly Pro Met Gly Asp Cys
225                 230                 235                 240
Gly Leu Thr Gly Arg Lys Ile Ile Val Asp Thr Tyr Gly Gly Met Ala
                245                 250                 255
Arg His Gly Gly Gly Ala Phe Ser Gly Lys Asp Pro Ser Lys Val Asp
            260                 265                 270
Arg Ser Ala Ala Tyr Ala Ala Arg Tyr Val Ala Lys Asn Ile Val Ala
            275                 280                 285
Ala Gly Leu Ala Asp Arg Cys Glu Ile Gln Val Ser Tyr Ala Ile Gly
            290                 295                 300
Val Ala Glu Pro Thr Ser Ile Met Val Glu Thr Phe Gly Thr Glu Lys
```

```
305                   310                   315                   320
Val Pro Ser Glu Gln Leu Thr Leu Leu Val Arg Glu Phe Phe Asp Leu
                  325                   330                   335
Arg Pro Tyr Gly Leu Ile Gln Met Leu Asp Leu Leu His Pro Ile Tyr
              340                   345                   350
Lys Glu Thr Ala Ala Tyr Gly His Phe Gly Arg Glu His Phe Pro Trp
          355                   360                   365
Glu Lys Thr Asp Lys Ala Gln Leu Leu Arg Asp Ala Ala Gly Leu Lys
      370                   375                   380
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<210> 19
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 19
ggaagcttaa gcagagatgc agagtgcg   28

<210> 20
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 20
ggaagcttgg tgcggtataa gaggccac   28

<210> 21
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 21

gggcatgctg tagtgaggta atcaggtt   28

<210> 22
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 22
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<210> 23
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 23
tgtctgctgg gcggtaca   18

<210> 24
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 24
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<210> 25
<211> 930
<212> DNA
<213> Escherichia coli

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<221> CDS
<222> (1)..(927)

<400> 25

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 1               5                  10                  15

gaa gaa aac gtc ttt gtg atg aca act tct cgt gcg tct ggt cag gaa    96
Glu Glu Asn Val Phe Val Met Thr Thr Ser Arg Ala Ser Gly Gln Glu
                20                  25                  30

att cgt cca ctt aag gtt ctg atc ctt aac ctg atg ccg aag aag att   144
Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile
            35                  40                  45

gaa act gaa aat cag ttt ctg cgc ctg ctt tca aac tca cct ttg cag   192
Glu Thr Glu Asn Gln Phe Leu Arg Leu Leu Ser Asn Ser Pro Leu Gln
        50                  55                  60

gtc gat att cag ctg ttg cgc atc gat tcc cgt gaa tcg cgc aac acg   240
Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr
65                  70                  75                  80

ccc gca gag cat ctg aac aac ttc tac tgt aac ttt gaa gat att cag   288
Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln
                85                  90                  95

gat cag aac ttt gac ggt ttg att gta act ggt gcg ccg ctg ggc ctg   336
Asp Gln Asn Phe Asp Gly Leu Ile Val Thr Gly Ala Pro Leu Gly Leu
            100                 105                 110

gtg gag ttt aat gat gtc gct tac tgg ccg cag atc aaa cag gtg ctg   384
Val Glu Phe Asn Asp Val Ala Tyr Trp Pro Gln Ile Lys Gln Val Leu
            115                 120                 125

gag tgg tcg aaa gat cac gtc acc tcg acg ctg ttt gtc tgc tgg gcg   432
Glu Trp Ser Lys Asp His Val Thr Ser Thr Leu Phe Val Cys Trp Ala
        130                 135                 140

gta cag gcc gcg ctc aat atc ctc tac ggc att cct aag caa act cgc   480
Val Gln Ala Ala Leu Asn Ile Leu Tyr Gly Ile Pro Lys Gln Thr Arg
145                 150                 155                 160

acc gaa aaa ctc tct ggc gtt tac gag cat cat att ctc cat cct cat   528
Thr Glu Lys Leu Ser Gly Val Tyr Glu His His Ile Leu His Pro His
                165                 170                 175

gcg ctt ctg acg cgt ggc ttt gat gat tca ttc ctg gca ccg cat tcg   576
Ala Leu Leu Thr Arg Gly Phe Asp Asp Ser Phe Leu Ala Pro His Ser
            180                 185                 190

cgc tat gct gac ttt ccg gca gcg ttg att cgt gat tac acc gat ctg   624
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Arg Tyr Ala Asp Phe Pro Ala Ala Leu Ile Arg Asp Tyr Thr Asp Leu
        195             200             205
gaa att ctg gca gag acg gaa gaa ggg gat gca tat ctg ttt gcc agt    672
Glu Ile Leu Ala Glu Thr Glu Glu Gly Asp Ala Tyr Leu Phe Ala Ser
    210             215             220
aaa gat aag cgc att gcc ttt gtg acg ggc cat ccc gaa tat gat gcg    720
Lys Asp Lys Arg Ile Ala Phe Val Thr Gly His Pro Glu Tyr Asp Ala
225             230             235             240
caa acg ctg gcg cag gaa ttt ttc cgc gat gtg gaa gcc gga cta gac    768
Gln Thr Leu Ala Gln Glu Phe Phe Arg Asp Val Glu Ala Gly Leu Asp
                245             250             255
ccg gat gta ccg tat aac tat ttc ccg cac aat gat ccg caa aat aca    816
Pro Asp Val Pro Tyr Asn Tyr Phe Pro His Asn Asp Pro Gln Asn Thr
                260             265             270
ccg cga gcg agc tgg cgt agt cac ggt aat tta ctg ttt acc aac tgg    864
Pro Arg Ala Ser Trp Arg Ser His Gly Asn Leu Leu Phe Thr Asn Trp
            275             280             285
ctc aac tat tac gtc tac cag atc acg cca tac gat cta cgg cac atg    912
Leu Asn Tyr Tyr Val Tyr Gln Ile Thr Pro Tyr Asp Leu Arg His Met
            290             295             300
aat cca acg ctg gat taa                                            930
Asn Pro Thr Leu Asp
305
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<210> 26
<211> 309
<212> PRT
<213> Escherichia coli

<400> 26

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 1               5               10              15
Glu Glu Asn Val Phe Val Met Thr Thr Ser Arg Ala Ser Gly Gln Glu
            20              25              30
Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile
        35              40              45
Glu Thr Glu Asn Gln Phe Leu Arg Leu Leu Ser Asn Ser Pro Leu Gln
        50              55              60
Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr
65              70              75              80
Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln
```

```
                        85                      90                      95
        Asp Gln Asn Phe Asp Gly Leu Ile Val Thr Gly Ala Pro Leu Gly Leu
                    100                     105                     110
        Val Glu Phe Asn Asp Val Ala Tyr Trp Pro Gln Ile Lys Gln Val Leu
                    115                     120                     125
        Glu Trp Ser Lys Asp His Val Thr Ser Thr Leu Phe Val Cys Trp Ala
                    130                     135                     140
        Val Gln Ala Ala Leu Asn Ile Leu Tyr Gly Ile Pro Lys Gln Thr Arg
        145                     150                     155                     160
        Thr Glu Lys Leu Ser Gly Val Tyr Glu His His Ile Leu His Pro His
                            165                     170                     175
        Ala Leu Leu Thr Arg Gly Phe Asp Asp Ser Phe Leu Ala Pro His Ser
                    180                     185                     190
        Arg Tyr Ala Asp Phe Pro Ala Ala Leu Ile Arg Asp Tyr Thr Asp Leu
                    195                     200                     205
        Glu Ile Leu Ala Glu Thr Glu Glu Gly Asp Ala Tyr Leu Phe Ala Ser
                    210                     215                     220
        Lys Asp Lys Arg Ile Ala Phe Val Thr Gly His Pro Glu Tyr Asp Ala
        225                     230                     235                     240
        Gln Thr Leu Ala Gln Glu Phe Phe Arg Asp Val Glu Ala Gly Leu Asp
                            245                     250                     255
        Pro Asp Val Pro Tyr Asn Tyr Phe Pro His Asn Asp Pro Gln Asn Thr
                    260                     265                     270
        Pro Arg Ala Ser Trp Arg Ser His Gly Asn Leu Leu Phe Thr Asn Trp
                    275                     280                     285
        Leu Asn Tyr Tyr Val Tyr Gln Ile Thr Pro Tyr Asp Leu Arg His Met
            290                     295                     300
        Asn Pro Thr Leu Asp
        305
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<210> 27

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 27

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<210> 28
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer
<400> 28
tagatcgtat agcgtgctct ggtagac   27

<210> 29
<211> 309
<212> PRT
<213> Escherichia coli

<400> 29
Ala Met Leu Pro Val
                    5

What is claimed is:

    1.   A microorganism which is deficient in repressor of L-methionine biosynthesis system and has L-

5 methionine productivity.

    2.   A microorganism having enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity.

10

    3.   A microorganism which is deficient in repressor of L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity.

15

    4.   The microorganism according to any one of claims 1 to 3, which further exhibits reduced intracellular S-adenosylmethionine synthetase activity.

20   5.   The microorganism according to any one of claims 2 to 4, wherein the enhanced homoserine transsuccinylase activity is obtained by increasing copy number of a gene coding for the intracellular homoserine transsuccinylase, or enhancing an expression regulatory

25 sequence for the gene.

    6.   The microorganism according to claim 1 or 4,

which has homoserine transsuccinylase for which
concerted inhibition by L-methionine and S-
adenosylmethionine is desensitized.

5   7.   The microorganism according to any one of
claims 1 to 6, which exhibits L-threonine auxotrophy.

8.   The microorganism according to any one of
claims 1 to 7, which exhibits enhanced intracellular
10 cystathionine γ-synthase activity and enhanced
intracellular aspartokinase-homoserine dehydrogenase II
activity.

9.   The microorganism according to any one of
15 claims 1 to 8, which belongs to the genus *Escherichia*.

10.   A method for producing L-methionine which
comprises culturing the microorganism according to any
one of claims 1 to 9 in a medium to produce and
20 accumulate L-methionine in the medium, and collecting
the L-arginine from the medium.

11.   A DNA which codes for homoserine
transsuccinylase for which concerted inhibition by L-
25 methionine and S-adenosylmethionine is desensitized,
wherein the homoserine transsuccinylase has the amino
acid sequence of SEQ ID NO: 26 including a mutation

corresponding to replacement of arginine by cysteine at

the 27th position, mutation corresponding to replacement

of isoleucine by serine at the 296th position, mutation

corresponding to replacement of proline by leucine at

5   the 298th position, mutation corresponding to

replacement of arginie by cysteine at the 27th position

and replacement of isoleucine by serine at the 296th

position, mutation corresponding to replacement of

isoleucine by serine at the 296th position and

10   replacement of proline by leucine at the 298th position,

mutation corresponding to replacement of proline by

leucine at the 298th position and replacement of

arginine by cysteine at the 27th position, or mutation

corresponding to replacement of arginine by cysteine at

15   the 27th position, replacement of isoleucine by serine

at the 296th position and replacement of proline by

leucine at the 298th position.

Abstract

L-Methionine is produced by culturing a

microorganism which is deficient in repressor of L-

5 methionine biosynthesis system and/or enhanced

intracellular homoserine transsuccinylase activity is

cultured in a medium so that L-methionine should be

produced and accumulated in the medium, and collecting

the L-methionine from the medium. The microorganism

10 preferably further exhibits reduced intracellular S-

adenosylmethionine synthetase activity, L-threonine

auxotrophy, enhanced intracellular cystathionine γ-

synthase activity and enhanced intracellular

aspartokinase-homoserine dehydrogenase II activity. The

15 present invention enables breeding of L-methionine-

producing bacteria, and L-methionine production by

fermentation.

Declaration, Power Of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION
_____

_____

the specification of which

     ☒ is attached hereto.

     ☐ was filed on _____ as

        Application Serial No. _____

        and amended on _____ .

     ☐ was filed as PCT international application

     Number _____

     on _____ ,

     and was amended under PCT Article 19

     on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed	
10-326717	Japan	17/11/1998	☒ Yes	☐ No
			☐ Yes	☐ No
			☐ Yes	☐ No
			☐ Yes	☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

_____ _____
(Application Number) (Filing Date)

_____ _____
(Application Number) (Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Yoshihiro USUDA
_____
NAME OF FIRST SOLE INVENTOR

Yoshihiro Usuda
_____
Signature of Inventor

November 2, 1999
_____
Date

Residence: Kawasaki-shi, Kanagawa, Japan
_____

_____

Citizen of: Japan
_____

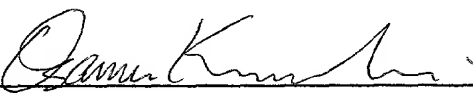Post Office Address: c/o Ajinomoto Co., Inc.,
Fermentation & Biotechnology Laboratories,
1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi,
Kanagawa, Japan

Osamu KURAHASHI

NAME OF SECOND JOINT INVENTOR

Signature of Inventor

November 2, 1999

Date

Residence: Kawasaki-shi, Kanagawa, Japan

Citizen of: Japan

Post Office Address: c/o Ajinomoto Co., Inc.,

Fermentation & Biotechnology Laboratories, 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, Japan

NAME OF THIRD JOINT INVENTOR

Signature of Inventor

Date

Residence: _____

Citizen of: _____

Post Office Address: _____

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

Date

Residence: _____

Citizen of: _____

Post Office Address: _____

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence: _____

Citizen of: _____

Post Office Address: _____